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PHYTOCHEMICAL EVALUATION AND METHOD VALIDATION FOR QUANTIFICATION OF FLAVONOIDS IN THE LEAVES OF *CINNAMOMUM VERUM*

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Abstract:

The activities reported for Cinnamomum verum may be associated with the presence of flavonoids and the quantification of these compounds has an important role in the evaluation of the quality of the species. The study aimed to validate a spectrophotometric methodology for the quantification of flavonoids in C. verum leaves. The extracts were obtained by reflux (0.25-1.25 g of the herbal drug; 40-80% ethanol) and the samples were analyzed after scanning (200-500 nm) and complexed with aluminum chloride (2.5-7.5%; $AlCl_3$) evaluated by reaction kinetics (0-60 min). Finally, the method was validated according to RDC 166/2017 of the National Health Surveillance Agency. The extraction was optimized using 0.5 g of the herbal drug and ethanol 80% (v/v) as a solvent, at 412 nm, 2.5% $AlCl_3$ with 30 min of reaction. The method was considered linear ($R^2 > 0.99$), with detection and quantification limits equal to 0.096 and 0.292 µg/mL, respectively. The specificity was evidenced by the parallelism between the curves obtained. In precision, a total flavonoid content of 1.25 g% (repeatability) and 1.19 g% (intermediate precision), expressed in rutin, was observed. The recovery values remained between 92-102% and the robustness presented a relative standard deviation < 5%. Thus, the analytical suitability of the method for the proposed purpose was demonstrated.

Keywords: *Cinnamomum verum. Phytochemical screening. Spectrophotometry. Validation.*

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1 INTRODUCTION:

The biodiversity can be appreciated for its ecological value, evolutionary role, and biological resource for popular medicine. The use of plants for medicinal purposes represents one of the oldest devices used by men to treat and/or cure diseases. And popular analyzes on the use and effectiveness of plants, support scientific research and contribute to the dissemination of the therapeutic characteristics of species [1].

Inserted in popular culture is the species *Cinnamomum verum* J. Presl. (synonym *Cinnamomum zeylanicum* Blume), which belongs to the Lauraceae family, native to Sri Lanka and tropical Asia. Named worldwide as "cinnamon", in Brazil it is known "Canela", "Canela-do-Ceilão" or "Canela-verdadeira" [2,3]. The species was the first aromatic plant used in the treatment of chronic bronchitis. Its use in popular medicine is due to the different therapeutic effects, such as stimulant, antidiabetic, antispasmodic, anti-inflammatory, among others [4,5]. These beneficial properties associated with the species have been attributed to a class of secondary metabolites, the phenolic compounds, more precisely those classified as flavonoids [6].

The flavonoids represent a class of molecules that are widely distributed throughout the Plantae and are the most diversified among products of natural origin [7]. Originating from the mixed route of shikimic acid and acetate, they present several properties of pharmacological interest that justify the use of plant species that present them in the composition, among which we can mention: antitumor, anti-inflammatory, antiviral, antioxidant activities, antimicrobial, anticancer [7-11].

Several techniques are described in the literature for the detection and quantification of flavonoids, highlighting the technique of absorption spectrophotometry in the ultraviolet-visible region (UV/Vis) due to its simplicity, speed, robustness, and low cost [12]. Even considering the advantages presented, the low selectivity is the main challenge of the spectrophotometric analysis of complex matrices, such as herbal extracts. The chemical structure of polyphenols allows absorption spectra depending on the number and position of the substituents, which can cause overlapping bands and limit the absorption of the compound of interest [13,14].

For the quantification of flavonoids without interference from other phenolic compounds present in the sample, the aluminum chloride complexation ($AlCl_3$) method is used, based on the ability of these molecules to form stable complexes in the presence of the Al^{3+} cation resulting in bathochromic displacement. Aluminum reacts only with the hydroxyl group of a portion in the B ring, making this technique specific for flavonoids. In this way, it avoids interference from other phenolic compounds, particularly phenolic acids, which invariably accompany flavonoids in plant tissues [12,15]. The reaction procedure with aluminum chloride also allows the estimation of the content of total flavonoids, due to the specificity for aglycone (free flavonoids) and glycosylated components, increasing the representativeness of the analysis and minimizing the possibility of deviations [16].

For safe use of complex matrices such as herbal drugs, it is necessary that standardization is established, demonstrating the authenticity of the raw material, as well as the content of its constituents, and for this, the validation of the analytical methodology is used [17]. The validation of analytical methods is an essential procedure for quality control and must ensure, through experimental studies, that the method meets the requirements of analytical applications, ensuring the reliability and reproducibility of the results obtained [18].

This work aimed to optimize and validate a spectrophotometric methodology in the ultraviolet-visible range for quantifying flavonoids in *Cinnamomum verum* leaves following the parameters established by Agência Nacional de Vigilância Sanitária (Anvisa), to ensure that the method in research has a consistent performance capability for the proposed purpose.

2 MATERIALS AND METHODS:

2.1 Herbal Material

The leaves of *C. verum* were collected in Recife-PE (8º02'49'' S and 34º56'56'' W). The identification was carried out in the herbarium Dárdano de Andrade-Lima at the Agronomic Institute of Pernambuco with the voucher 91609 and the registration carried out in SisGen (A10D0F2). Then, the herbal material was dried in an oven for 7 days at 40 ºC (LUCA-82-480, Lucadema®) and grounded in a knife mill (TE-680, Tecnal®) for subsequent analyzes.

2.2 Phytochemical Analysis

About 1 g of the dried and ground herbal material was transferred to a round bottom flask and 10 mL of methanol (P.A., Dinâmica®) were added. The solution was kept under reflux for 15 min under 85 ºC in a water bath (LUCA-150/24/D; Lucadema®) and, finally, cooled and filtered. From the filtrate, 10 µL aliquots were removed and applied to silica gel plates (Art. 1.05554.0001, Merck®) for evaluation by Thin Layer Chromatography (TLC). The chromatograms were obtained using the elution systems and standards for the respective classes of

metabolites described in Table 1, adapted [19]. After elution, the plates were dried and observed under white light; then they were derivatized and observed at 254 and 365 nm wavelengths.

Table 1. Chromatographic systems, reagents, and standards used to obtain the phytochemical profile of leaves C*innamomum verum*.

Metabolites	System of elution	Reagents	Standards
Alcaloids	AcOEt:HCOOH:AcOH:H_2O (100:11:11:26)	Dragendorff	Pilocarpine
Coumarin	EtOEt:Toluen:AcOH10% (50:50:50)	KOH + UV	Coumarin
Cinnamic acids	AcOEt:HCOOH:H_2O (90:5:5)	NEU + PEG	Caffeic acid
Flavonoids			Rutin
Hydrolysable Tannins		$FeCl_3$	Gallic acid
Condensed Tannins		VC	Catechin
Terpenes/Steroids	Toluen:AcOEt (70:30)	LB	β-sitosterol

AcOEt: Ethyl acetate; HCOOH: Formic acid, H_2O: Water, AcOH: Acetic acid, EtOEt: Ethyl ether; NEU: 1% ethyl-boryl aminoester acid in methanol; PEG: Propylene glycol; UV: Ultraviolet; KOH: Potassium hydroxide; VC: Hydrochloric Vanillin; LB: Lieberman-Burchard; $FeCl_3$: ferric chloride.

2.3 Evaluation of the method for quantification of flavonoids

Preparation of the extractive solution

The extractive solution was obtained under reflux in a round-bottomed flask using 0.5 g of herbal drug and 30 mL of 80% (v/v) ethanol, proceeding with extraction for 30 min in a water bath at 85 ± 2 °C. Then, the solution was cooled to room temperature and filtered through cotton, with the residue (cotton and herbal drug) being extracted twice more, for 10 min. The filtered fractions were combined in a volumetric flask and the volume was adjusted to 100.0 mL with the same solvent, constituting the Stock Solution (SS).

Solvent concentration

The SS were prepared with different concentrations of the 40%, 60%, and 80% (v/v) ethanolic solution. From the different extracts obtained, aliquots of 8 mL were transferred to 25 mL volumetric flasks, where 1.0 mL of methanolic aluminum chloride solution (2.5% $AlCl_3$; w/v) was added. Then, the volume was adjusted with the respective extraction solvent. The absorbance was measured on a spectrophotometer (Evolution 60S; Thermo Scientific®) after 30 min of reaction. The blank solution being prepared in the same way as the sample, without adding the $AlCl_3$ reagent (2.5%; w/v).

Amount of drug and wavelength of analysis

Extractive solutions prepared with different amounts of herbal drug were evaluated: 0.25; 0.50; 0.75; 1.00 and 1.25 g, to verify the influence of the herbal drug/solvent (w/v) ratio on the response of the method.

For the determination of the wavelength that presented the most adequate absorbance value for the method, the samples were subjected to scanning in a spectrophotometer in the range of 200 to 500 nm.

Aliquot of analysis and Aluminum chloride

From the SS obtained with the best drug/solvent ratio, different aliquots were transferred to a 25 mL volumetric flask, with the addition of 1.0 mL of $AlCl_3$ and adjusting the volume with the solvent. Aliquots of 5.0; 6.0; 7.0 and 8.0 mL of the SS were tested in triplicate. For the $AlCl_3$ aliquots, the following were tested: 1.0; 2.0 and 3.0 mL, maintaining the reaction time of 30 min followed by scanning in a spectrophotometer.

Evaluation of reaction kinetics

Reaction kinetics evaluated the effects of reaction time and $AlCl_3$ concentration on the response of the method. Dilutions were made from the predetermined SS, using different concentrations of $AlCl_3$: 2.5; 5.0 and 7.5% (w/v), followed by analysis on a spectrophotometer at specific intervals of: 0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55 and 60 min after adding the $AlCl_3$ methanolic solution.

All experiments were carried out in triplicate and expressed as mean ± standard deviation (sd) (relative standard deviation; RSD%) and calculated as rutin (g% of the dry herbal drug).

2.4 Validation

The validation tests were performed according to the spectrophotometric conditions determined during the method optimization study. All validation parameters followed the Guide for Validation of Analytical and Bioanalytical Methods, available in RDC 166 [18] and included tests of linearity, limits of detection and quantification, specificity, precision, accuracy, and robustness. The standard used in the tests was rutin (> 96%, Sigma-Aldrich®) and all analyzes were performed in triplicate. The results were determined by the mean ± sd and RSD%; and, analysis of variance (ANOVA), Student's t-test and F test were used, when appropriate. The significance limit considered $p < 0.05$.

Linearity
To evaluate the linearity of the sample and the standard, the linear regression calculation was used by the least-squares method, based on calibration curves using six sample concentrations (0.8-2.8 mg/mL) and the standard (0.6-1.6 mg/mL). The results allowed to establish the coefficient of determination, using $R^2 > 0.99$ as the minimum acceptable value and the equation obtained.

Limits of detection and quantification
The limits of detection (LD) and quantification (LQ) were estimated from the regression data of the standard analytical curves according to RDC 166.

Specificity
Specificity was obtained by the standard addition method, where 3.0 mL of the rutin solution (0.6 mg/mL) was added to each of the six concentrations used in the linearity, followed by the addition of 1.0 mL of $AlCl_3$ (2.5%, w/v) with 30 min of reaction, aiming to demonstrate the parallelism between the sample and sample added with the standard curves.

Precision
The precision was performed by the repeatability (intra-run precision) and intermediate precision (inter-run precision) analyzes. Repeatability was established, in a single day and single analyst, by analyzing six samples of the 100% extractive solution with the subsequent addition of 1.0 mL of $AlCl_3$ (2.5%, w/v) followed by 30 min of reaction. The intermediate precision was defined on two consecutive days, by two different analysts. The results were evaluated considering the RSD, Student's t-test, and F test.

Accuracy
Accuracy was performed by recovery tests where known amounts of the standard rutin solution (0.6, 1.0, and 1.6 mg/mL) were added to the 100% concentration sample solution. The analyzes were performed in triplicate for each concentration level, making it possible to calculate the recovery values, expressed as a percentage. The method must present a recovery percentage between 85 and 115%.

Robustness
The robustness of the method was assessed through intentional changes in experimental procedures, including variation in luminosity (presence and absence of light), the stability of the extractive solution (analysis on day 1 and day 3 after preparation of the extractive solution), different manufacturers of the solvent absolute ethyl alcohol (Exôdo® and Química Moderna®) and use of different spectrophotometers (Micronal® and Thermo Scientific®). The results were analyzed from mean ± sd and RSD, Student's t-test was also used.

3. RESULTS AND DISCUSSION:

The herbal drug was characterized by TLC, through the manual application of the samples. The results of the analysis indicated the presence of groups of substances of secondary metabolism in the herbal material such as alkaloids, cinnamic derivatives, flavonoids, condensed tannins, hydrolysable tannins, terpenes, and steroids.
The presence of gallic acid (blue-gray color, Rf = 0.18), rutin (orange color, Rf = 0.32), caffeic acid (fluorescent blue color, Rf = 0.21), catechin (pink-red color; Rf = 0.73) and β-sitosterol (fluorescent bluish-white color, Rf = 0.23) were evidenced in the raw material, showing spots of the same color and Rf similar to the standards used. Other fluorescent yellow and orange spots were also found, characteristic of the flavonoid class with values and Rf equal to 0.43; 0.51; 0.62; 0.76; 0.81; 0.91. Orange spots were also observed after derivatization with Dragendorff's reagent (with Rfs equal to 0.80; 0.87; 0.97), however, none corresponded to the Rf (0.50) of the standard used. The negative result for coumarins is justified by the absence of the fluorescent green characteristic of this metabolite.
The preliminary phytochemical analysis aims to identify the chemical components present in the plant, arising from its secondary metabolism. These metabolites serve as markers for the species under study, or even the region in which the plant was found. The phytochemical profile of herbal material, one can objectively plan the best methods for extraction and bioassays for later isolation of the active ingredient. Thus, this analysis is fundamental when there are no chemical studies on the species of interest, being useful to analyze the quality of the herbal drug and offer useful data to trace the phylogenetic profile of the herbal materials [20]. The results found are following the study by Gomes, Pena, and Almeida (2016) [21], where the presence of alkaloids, phenols, tannins, and flavonoids in *C. verum* leaf extracts was also evidenced. The study in question worked with extracts obtained by percolation using methanol and additionally the presence of saponins and anthraquinones was identified.

For the quantification of flavonoids through direct dilution method, extractions are performed with hydroalcoholic solutions testing different proportions of ethanol. In this step, the best proportion of solvent among those proposed is verified and the best responses were obtained with 80% ethanol, resulting in average levels of 1.53 g% ± 0.0268 (1.74%) expressed in rutin (Figure 1).

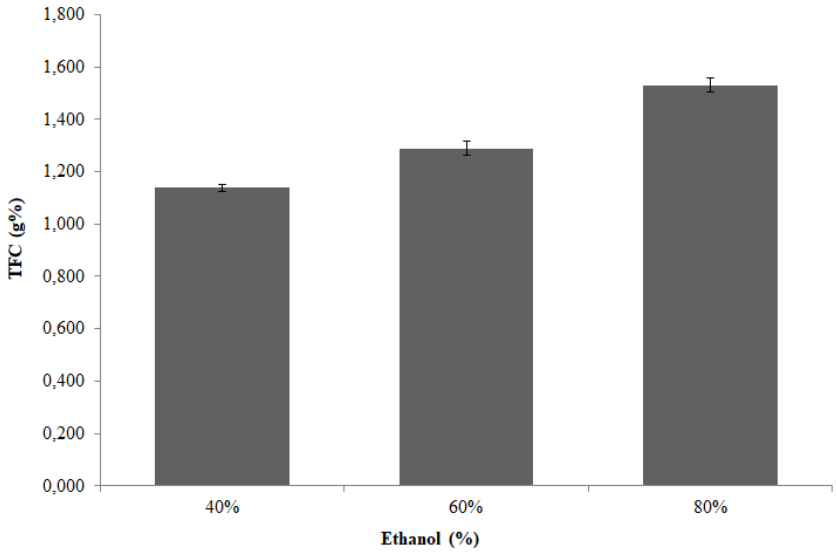

Figure 1. Total flavonoids content (TFC) obtained with different concentrations of ethanol.

The concentration and type of solvent are fundamental aspects of this type of procedure. The conditions for the extraction must not only be exhaustive but also selective and the structures of the flavonoids influence the extractive behavior in hydroalcoholic solutions. The presence, position, and several hydroxyls result in an influence on the UV-Vis spectrum [22].

Regarding the amount of herbal material used, different quantities were tested (0.25 - 1.25 g), the results showed greater analytical performance for the 0.50 g and 0.75 g samples. However, the one that obtained the best yield in g% of rutin was 0.50 g, with an average content equal to 1.45 g% ± 0.0207 (1.42%) (Figure 2). The use of samples with larger amounts of drug (1.00 - 1.25 g) showed a decrease in yield, with lower values of total flavonoid content, demonstrating that there may have been a saturation of the extractor solvent combined with a depletion of the reagent.

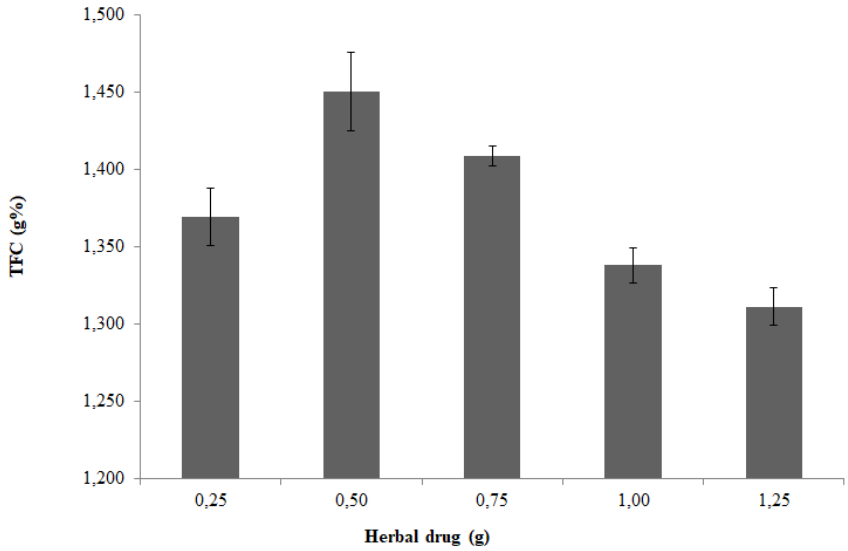

Figure 2. Total flavonoids content (TFC) obtained with different proportions of herbal drug.

Establishing the maximum absorption wavelength is of fundamental importance in the quantification of flavonoids since the observed maximums can provide information about the substances that are contained in the sample. Considering that flavonoids can be classified as aglycone and/or heterosides, the location of the chromophore can hide details related to the structure of these metabolites, making it necessary to employ complexation reactions that promote displacements in the maximum absorption wavelength that may be present, linked to the substitution patterns and/or degree of hydroxylation of the molecules, increasing the specificity of the analysis [12,22]. Therefore, the most appropriate wavelength for the method was set at 412 nm, with maximum absorption.

After determining the wavelength, the appropriate dilution for analysis was established, using different aliquots from the stock solution obtained from the herbal material. To obtain working concentrations capable of providing absorbances between 0.2 to 0.8 U.A. (Units of Absorbance), based on the Lambert-Beer law, the working rate was set at 8 mL, resulting in absorbance of 0.430 ± 0.0062 (1.45%).

For the different $AlCl_3$ reagent aliquots tested it was possible to observe very similar results of absorbance, where: 1 mL showed values of 0.433 ± 0.0035 (0.81%), 2 mL: 0.430 ± 0.0062 (1.45%), 3 mL: 0.437 ± 0.0119 (2.73%). The rates do not show statistically significant differences, at a 95% confidence level, from the Student's t-test, where the calculated t values (t_{1-2}: 0.80; t_{2-3}: 0.94; t_{1-3}: 0.55) remained lower than the tabulated t (2.13). Thus, the 1 mL aliquot was selected, indicating that the reagent concentration resulting from this aliquot can satisfy the total flavonoids present in the solution and forming the flavonoid-Al^{3+} complexes.

The time required for the formation of flavonoid-Al^{3+} complexes is defined as reaction time and depends on factors, such as the pattern of aglycone replacement. It is important to emphasize that the profile of complex formation is different when using herbal extracts and when using pure substances. In this way, evaluating and establishing the time for these complexes to be formed, and their stability after they are formed is a crucial step to avoid erroneous data later. Figure 3 shows the kinetics of complexation of the solution from leaves of *C. verum* with different concentrations of aluminum chloride for 60 min. It was verified that the maximum absorbance of the samples was reached at 30 min and that the reaction product remained stable until the end of the experiment. Considering the influence of the concentration of $AlCl_3$, the profiles of complex formation was similar for all cases, establishing the lowest concentration of $AlCl_3$ 2.5%, as the ideal one, since, there is a report in the literature that concentrations high levels of aluminum chloride can promote the reversion of the complex formed.

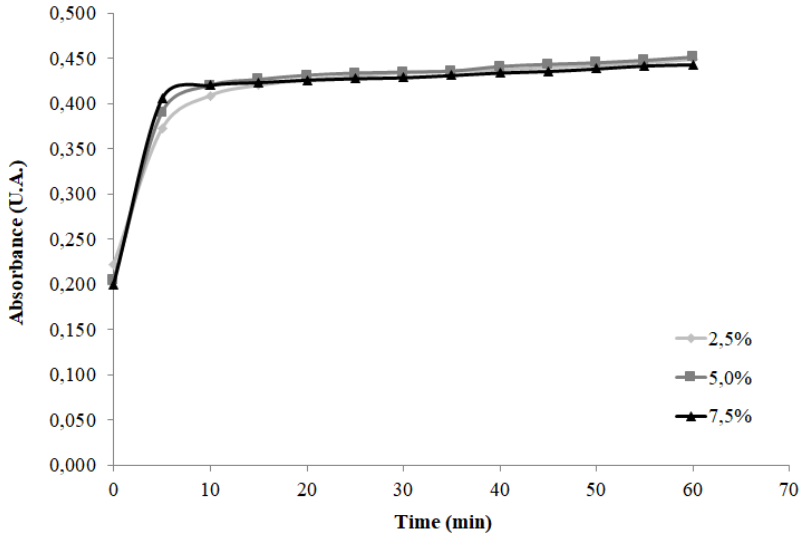

Figure 3. Complexation kinetics of flavonoid with aluminum chloride.

With the conditions of *C. verum* leaves extracts optimized in 0.5 g of herbal drug, 80% ethanol (v/v), 1 mL of 2.5% AlC_3 (w/v), wavelength 412 nm after 30 min of reaction, the method was then validated as recommended by Anvisa.

Linearity can be defined as the ability of the method to obtain responses directly proportional to the presence of the analyte in the sample and presents itself as an important test in the validation of analytical methods because from the generated calibration curves, it is possible to determine the relationship between the response instrumental and the concentration of the analyte in the study ranges [18]. The results obtained from the linear regression analysis proved that the substances show linearity in the studied concentration ranges, as recommended by the legislation (80-120%), with determination coefficients greater than 0.99. That is, more than 99% of the data can be explained by the models obtained in the methodologies, indicating a linear relationship between the increase in the concentration of the analyte and the spectrophotometric response, with the calibration curves of standard ($y = 0.4947x - 0.071$; $R^2 = 0.9986$) and sample ($y = 0.2506x + 0.0358$; $R^2 = 0.9988$).

The Limit of Detection (LD) and Limit of Quantification (LQ) calculated were 0.096 and

0.292 µg/mL, respectively. With these results, we can see that the method has a sensitivity to detect and quantify the total flavonoids in the extracts of *C. verum* without interference from the equipment.

Specificity assesses the method's ability to detect the substance of interest in the presence of other substances. For this test, the standard addition method was used and the results obtained show the parallelism between the lines (linearity and specificity), proving the capacity of the method to detect the substance of interest in the presence of others substances. Also, the similarity between the slope coefficients obtained shows the efficiency of the methodology in obtaining answers for the substances of interest, indicating the absence of interference from other constituents of the analyzed herbal matrix.

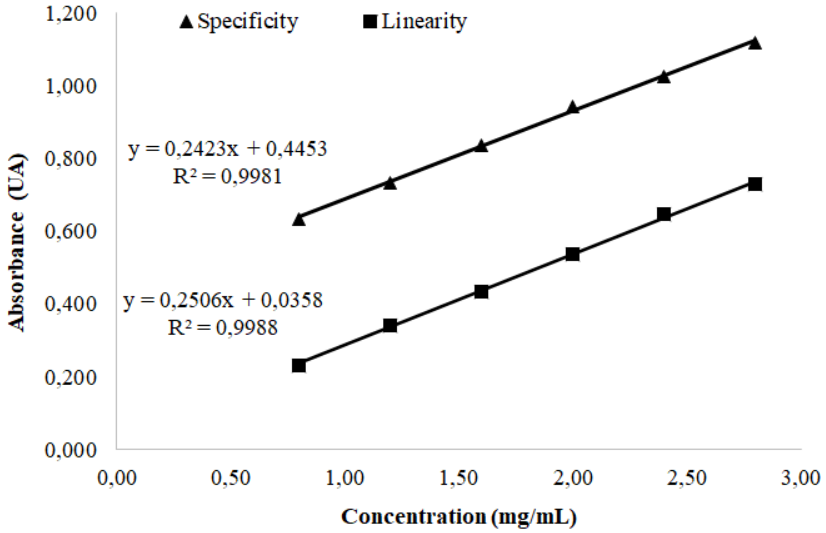

Figure 4. Specificity and linearity curves.

Precision when assessed at the repeatability level, in which the answers were obtained by the same analyst on the same day, obtained a total flavonoid content equal to 1.25 g% ± 0.0375 (2.98%), the variability observed through the RSD < 5%, within the limit required by the legislation (maximum of 5%). In the intermediate precision, the conformities between the results obtained by different analysts on different days were evaluated. For this analysis, the legislation also determines that RSD values are below 5%. Thus, the data found to allow us to state that the proposed method for quantification of flavonoids can be considered precise since it meets the requirements determined by RDC 166, as can be seen in Table 2. The statistical analysis, for intermediate precision, by the Student t-test, for the evaluation of the different analysts and by the F test, for the different days, demonstrated that the calculated t/F values were lower than the t/F tabulated, indicating that there is no significant difference between the samples, at a level of 95% reliable.

Table 2. Results of Total Flavonoids Content (TFC) for intermediate precision.

Days	Analyst	TFC (g%)	Test *t*	Test F
1	1	1.22 ± 0.0120 (0.98%)	t_{cal}: 1.10	
	2	1.17 ± 0.0104 (0.55%)	t: 4.30	F_{cal}: 2.85
2	1	1.22 ± 0.0067 (0.58%)	t_{cal}: 1.12	F: 5.05
	2	1.16 ± 0.0334 (0.27%)	t: 4.30	

According to Anvisa, the accuracy of an analytical method is the proximity of the experimental results obtained by the method under study concerning the true value. To determine the accuracy of the analytical methodology developed and evaluate whether the results reflect the actual values present

in the samples, the addition of the standard in the sample was used and the accuracy was calculated from the recovery of the total content searched in the proposed methodology. Recovery values of 92 to 102% were obtained and these percentages are within the range of 85-115% recommended by current legislation. In addition, RSD was less than 5% at all concentration levels assessed, low (80%): 0.24%; medium (100%): 0.18% and high (120%): 1.44%. From these results, we can conclude that the proposed quantitative procedures are following the requirements established by the legislation, offering reliable data, especially when considering the complex nature of the herbal matrix.

Finally, small, and deliberate variations were made in the methodology to assess the robustness of the method. The parameters evaluated included: different solvent manufacturers; spectrophotometric analysis on different days; the presence and absence of light; and analysis on different equipment. All the changes made did not significantly affect the results of the levels found, with RSD less than 5%, indicating that the proposed method was able to resist the different experimental conditions. For statistical analysis, Student's t-test was used to analyze the variation within the groups, showing values for: different solvent manufacturers: t_{cal} = 0.55; stability of the extractive solution: t_{cal} = 0.05; brightness variation: t_{cal} = 2.80; different equipment: t_{cal} = 0.48; considering t_{tab} value = 2.91. The results showed that there is no significant difference ($p < 0.05$), since the calculated t values (t_{cal}) were lower than the tabulated t values (t_{tab}), showing the robustness of the method.

5 CONCLUSIONS:

The spectrophotometric methodology proposed in this study, for the quantification of total flavonoids in the extract of *C. verum* leaves, proved to be effective for the proposed purpose. The analytical responses obtained during the optimization of the method revealed the suitability of the procedure for quantification. The proposed method was linear, specific, accurate, precise, and robust, under the different experimental conditions studied, as described in this work. Thus, following the requirements for analytical use and to ensure the reliability of the results, the method is suitable for use as a quality control tool.

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